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Human 2-5A synthetase: characterization of a novel cDNA and corresponding gene structure

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The enzyme 2-5A synthetase is induced in cultured cells in response to interferon (IFN) treatment. A λgt10 cDNA library of mRNA from IFN-induced Daudi lymphoblastoid cells was screened with oligonucleotide probes. Several overlapping cDNAs were isolated and shown to be derived from the human synthetase gene using filter selection and oocyte microinjection assays. The nucleotide sequence of one of these, cDNA 8-2, extended the 2-5A synthetase sequence already described 72 bp in the 5′ direction but was found to differ significantly in coding sequence at the 3′ end. The longest cDNA isolated (6-2) was ~1.4 kb. By Northern hybridization analysis single mRNAs of 1.7 kb were detected in Daudi and T98G (glioblastoma) cells. However, in HeLa cells, four mRNAs ranging in size from 1.5 to 3.5 kb were found, one of which differed at the 3′ end. Analysis of both phage and cosmid genomic clones and comparison with genomic DNA indicate that there is a single gene for 2-5A synthetase, comprising at least six exons and five introns, which can undergo a novel form of alternative RNA processing depending on cell type.

Key words: 2-5A synthetase/cDNA/genomic structure/differential splicing/interferon

Introduction

The interferons (IFN) are a family of host-range-specific proteins produced by animals and cultured cells in response to viral infection or to other inducers such as double-stranded RNA (dsRNA). One of a number of proteins induced in cells by IFN treatment is the enzyme 2-5A synthetase (reviewed in Williams, 1983). This enzyme is thought to play a role in the antiviral action of IFN through the synthesis of 2′-5′ linked oligomers of adenosine which activate a latent endoribonuclease which degrades mRNA (Clemens and Williams, 1978). As a result, protein synthesis and virus growth are inhibited. The 2-5A is rapidly degraded by a 2′-phosphodiesterase so that activation of the nuclease and inhibition of protein synthesis are transient (Williams and Kerr, 1979; Williams *et al.*, 1978).

The 2-5A system may, however, be involved in more general aspects of cell growth and development which are independent of IFN. Basal levels of 2-5A synthetase activity vary widely in different cell lines, can be altered in response to hormone treatment (Cayley *et al.*, 1982) and do not always correlate with the ability of cells to support virus replication (Johnston and Torrence, 1984). In addition, the 2-5A-dependent endonuclease displays little substrate specificity during viral infection in that both host and viral mRNAs are degraded (Wreschner *et al.*, 1981). Furthermore, the endonuclease appears to be regulated independently of the IFN response, since, in a comparison of differentiated and undifferentiated teratocarcinoma cells, enzyme activity was detected only in the differentiated cells (Krause *et al.*, 1985).

As a first step towards investigating at a molecular level the induction of the 2-5A synthetase gene by IFN and the expression of this gene in a variety of growth and differentiation situations, we have isolated and characterized partial cDNAs for the 2-5A synthetase. Northern hybridization analysis showed that the size and number of mRNAs for 2-5A synthetase varied with the cell type examined. Analysis of genomic phage and cosmid clones and comparison with genomic DNA by Southern blot analysis revealed a single chromosomal gene for 2-5A synthetase which may undergo alternative RNA processing in some cultured cell lines.

Results

Screening of the Daudi cDNA library

Human lymphoblastoid Daudi cells are known to express high levels (~0.1% of total cell protein) of 2-5A synthetase activity which increase 3–5 times upon induction by IFN (Silverman *et al.*, 1982). In addition, these cells require as little as 1 u/ml IFN to exhibit the characteristic anti-growth effects (Gewert *et al.*, 1981). For these reasons, we screened a λgt10 library of IFN-induced cDNAs constructed using mRNA from Daudi cells.

To isolate cDNAs for the 2-5A synthetase, a total of 22 500 plaques were screened using eight end-labelled 20-mer oligonucleotide probes derived from the partial amino acid sequence described by Chebath *et al.* (1983). The estimated melting temperature of the oligonucleotide-cDNA hybrid was 48°C. Consequently, hybridizations were carried out at 37°C and the most stringent wash was at 47°C. Five phage isolates hybridized strongly to the oligonucleotide probes, the longest being 872 bp (cDNA 8-2). This clone was subsequently used to re-screen the library, resulting in the isolation of an additional cDNA (6-2) of 1397 bp. The cDNA clones 8-2, 6-2 and a short cDNA, 5-2, were selected for further characterization.

Filter selection

To demonstrate that the cDNAs represented the 2-5A synthetase, filter selection experiments were carried out. The plasmid pMS 8-2 was constructed by subcloning cDNA 8-2 into the *Eco*RI site of pUC 9. The mRNA used for selection was prepared from T98G cells induced with Wellferon for 8 h. This human cell line shows an ~20-fold induction of 2-5A synthetase activity in response to IFN (DeMaeyer *et al.*, 1982). Poly(A)$^+$ RNA selected by either pMS 8-2 or pUC 9 (negative control) was microinjected into oocytes and the extracts assayed for 2-5A synthetase activity. In this system injection of mRNA from uninduced T98G cells results in no detectable enzyme product. As shown in Figure 1, mRNA resulting in a product which corresponded to dimer 2-5A was eluted from the filter containing the cDNA 8-2 but remained in the unbound mRNA pool when

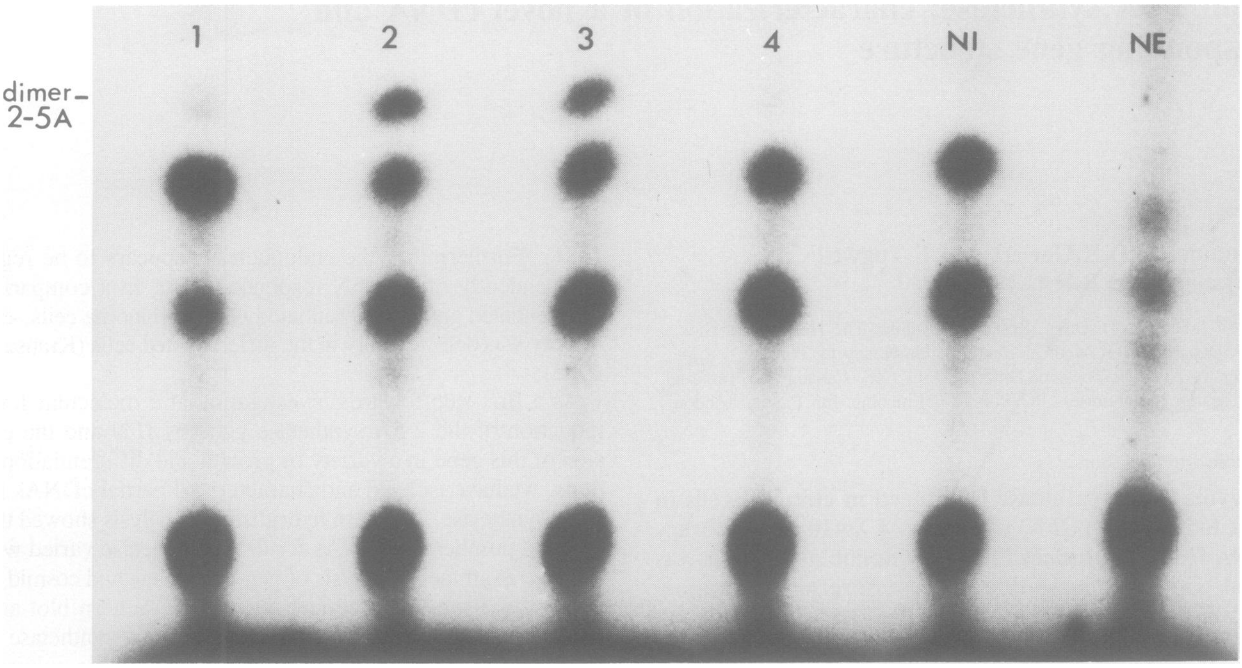


Fig. 1. Filter selection of mRNA from IFN-induced cells by cDNA 8-2. Plasmids pMS8-2 and control pUC 9 were bound to nitrocellulose filters and hybridized to poly(A)$^+$ RNA from induced T98G cells. Selected mRNAs were eluted and injected into oocytes for assay for 2-5A synthetase activity. The [^{32}P]ATP-labelled products were resolved by PEI-cellulose t.l.c., followed by autoradiography (see Materials and methods). Controls for uninjected oocyte (NI) and no extract (NE), background due to the [^{32}P]ATP substrate alone, are included. The position of the A2′p5′A (dimer 2-5A) marker is indicated. **Lane 1**, mRNA eluted from filter containing control plasmid pUC 9; **lane 2,** poly(A)$^+$ RNA remaining in the unbound pool after filter selection by pUC 9; **lane 3**, mRNA eluted from the pMS 8-2 filter; **lane 4**, remaining poly(A)$^+$ pool not bound by filter 8-2.

pUC 9 was used. This result shows that cDNA 8-2 is indeed derived from the 2-5A synthetase gene.

Restriction mapping and sequence analysis

Restriction maps of the cDNAs were constructed by standard endonuclease digestions (Figure 2a). The cDNA 6-2 (sequence available on request) is identical to cDNA 8-2 at the 3′ end and extends the 8-2 sequence 520 bp in the 5′ direction. The cDNA 5-2 contains 375 bp of the 3′ end of cDNA 8-2. Sequence analysis of fragments of cDNA 8-2 (or cDNA 5-2) was carried out by the dideoxy-M13 method (Sanger *et al.*, 1980) and is shown in Figure 2b. The restriction map and sequence of the cDNA clone E1 described by Merlin *et al.* (1983) is shown for comparison.

The sequence of cDNA 8-2 is identical to that of E1 from nucleotide 1 in the latter to nucleotide 278 and extends the 2-5A synthetase sequence 72 nucleotides in the 5′ direction (Figure 2b). However, from nucleotide 350 in cDNA 8-2 to the 3′ end, the restriction maps and sequences are completely different. The point of divergence from E1 has been confirmed in two other independent clones from the Daudi library (not shown) and no overlaps with the 3′ end of E1 are present in any of these clones. The total open reading frame in 8-2 consists of 687 nucleotide (229 amino acids). Two poly(A) addition signals are found, starting at nucleotides 841 and 847, which are followed by a short poly(A) tail starting at residue 862. Thus the 8-2 clone is a partial cDNA derived from a 2-5A synthetase gene with a novel nucleotide sequence at its 3′ end.

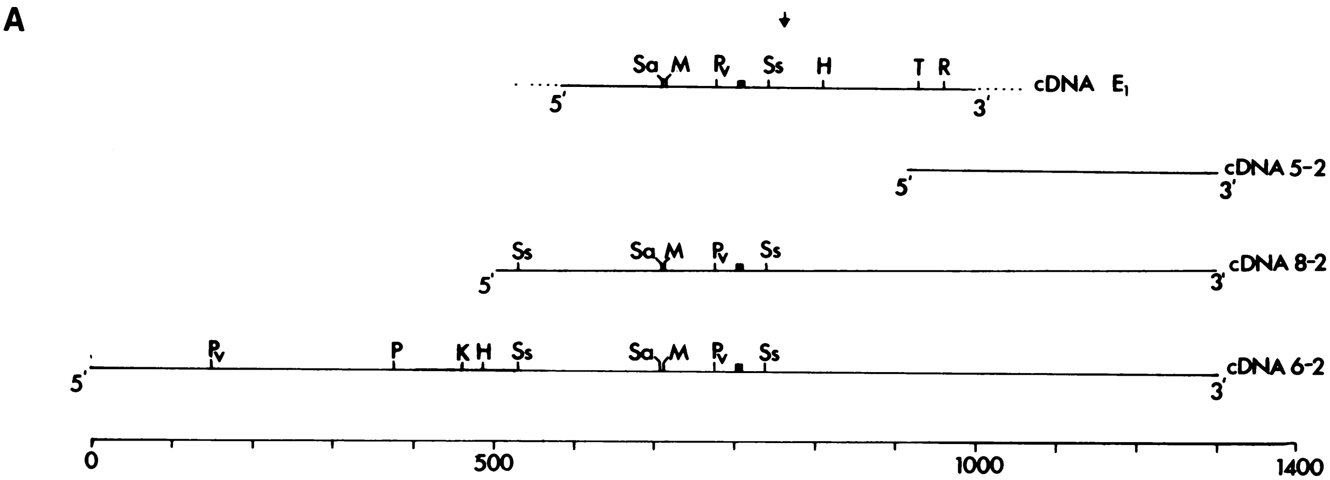
Analysis of 2-5A synthetase mRNA in T98G, Daudi and HeLa cells

Northern blot analysis was carried out on mRNA from T98G, Daudi and HeLa cells to determine the size of transcripts identified by the 8-2 homologous cDNAs. 10 μg of total RNA or 1.5 μg of poly(A)$^+$ RNA prepared from both control cells or cells induced with IFN were electrophoresed on denaturing formaldehyde agarose gels, blotted and hybridized to the nick-translated *Eco*RI inserts of cDNAs 8-2, 6-2 or 5-2. When Daudi and T98G cells were treated with IFN (T98G: 8 h, 5000 U/ml Wellferon; Daudi: 21 h, 500 U/ml Wellferon), mRNAs of the same size, 17S (1.7 kb), hybridized to cDNA 8-2 (Figure 3). Identical results (not shown) were found when either cDNA 6-2 or 5-2 was used as the probe. Daudi cells have a high basal level of 2-5A synthetase activity, which is reflected in the weak hybridization signal detected when mRNA from uninduced Daudi cells is examined. The dramatic increase in the amount of 2-5A synthetase mRNA in induced T98G cells correlated with the observed increase in enzyme activity (unpublished data). Sedimentation of T98G or Daudi poly(A)$^+$ RNA on sucrose gradients followed by oocyte translation of individual fractions showed that a mRNA of size 17S codes for 2-5A synthetase activity (data not shown). When the RNA fractions were analysed by Northern blot, only mRNA in the 17S fraction (1.7 kb) hybridized to cDNAs 6-2, 8-2 and 5-2 (not shown).

A time course experiment was done to determine the kinetics of mRNA induction in T98G cells (Figure 3, tracks 5-10). Cells were induced with Wellferon (2500 U/ml) for the indicated periods and total RNA prepared from each. Hybridization of Northern blots to cDNA 8-2 showed that mRNA levels increase from undetectable to peak at 6 h and then decrease by 24 h of induction. In Daudi cells peak synthetase mRNA accumulation does not occur until 24 h of IFN treatment (not shown).

The hybridization pattern of mRNA from HeLa cells is strikingly different from that seen in Daudi or T98G cells. In HeLa cells, four mRNA species of sizes ~3.5, 2.5, 1.7 and 1.5 kb are induced by treatment with IFN. These transcripts are maximally detected (Figure 4, lanes 3 and 4) in RNA prepared from HeLa cells treated for 10 h with either IFN-α2 or Wellferon.

A



B

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                       Sst I
cDNA 8-2 5' GCC CTG GAG CTC CTG ACG GTC TAT GCT TGG GAG CGA GGG AGC ATG AAA ACA CAT TTC 57
translation 8-2 ALA LEU GLU LEU LEU THR VAL TYR ALA TRP GLU ARG GLY SER MET LYS THR HIS PHE

cDNA E1             TTT CGG ACG GTC TTG GAA TTA GTC ATA AAC TAC CAG CAA CTC TGC ATC
AAC ACA GCC CAG GGA TTT CGG ACG GTC TTG GAA TTA GTC ATA AAC TAC CAG CAA CTC TGC ATC 120
ASN THR ALA GLN GLY PHE ARG THR VAL LEU GLU LEU VAL ILE ASN TYR GLN GLN LEU CYS ILE

TAC TGG ACA AAG TAT TAT GAC TTT AAA AAC CCC ATT ATT GAA AAG TAC CTG AGA AGG CAG CTC
TAC TGG ACA AAG TAT TAT GAC TTT AAA AAC CCC ATT ATT GAA AAG TAC CTG AGA AGG CAG CTC 183
TYR TRP THR LYS TYR TYR ASP PHE LYS ASN PRO ILE ILE GLU LYS TYR LEU ARG ARG GLN LEU

ACG AAA CCC AGG CCT GTG ATC CTG GAC CCG GCG GAC CCT ACA GGA AAC TTG GGT GGT GGA GAC
ACG AAA CCC AGG CCT GTG ATC CTG GAC CCG GCG GAC CCT ACA GGA AAC TTG GGT GGT GGA GAC 246
THR LYS PRO ARG PRO VAL ILE LEU ASP PRO ALA ASP PRO THR GLY ASN LEU GLY GLY GLY ASP

                    Pvu II
CCA AAG GGT TGG AGG CAG CTG GCA CAA GAG GCT GAG GCC TGG CTG AAT TAC CCA TGC TTT AAG
CCA AAG GGT TGG AGG CAG CTG GCA CAA GAG GCT GAG GCC TGG CTG AAT TAC CCA TGC TTT AAG 309
PRO LYS GLY TRP ARG GLN LEU ALA GLN GLU ALA GLU ALA TRP LEU ASN TYR PRO CYS PHE LYS

                            Sst I            ▼
AAT TGG GAT GGG TCC CCA GTG AGC TCC TGG ATT CTG CTG GTG AGA CCT CCT GCT TCC TCC CTG
AAT TGG GAT GGG TCC CCA GTG AGC TCC TGG ATT CTG CTG GCT GAA AGA ACA GTA CAG ACG ATG 372
ASN TRP ASP GLY SER PRO VAL SER SER TRP ILE LEU LEU ALA GLU ARG THR VAL GLN THR MET
   probe                               ...
CCA TTC ATC CCT GCC CCT CTC CAT GAA GCT TGA GAC ATA TAG CTG GAG ACC ATT CTT TCC AAA
AGA CCG ACG ATC CCA GGA CGT ATC AGA AAT ATG GTT ACA TTG GAA CAC ATG AGT ACC CTC ATT 435
ARG PRO THR ILE PRO GLY ARG ILE ARG ASN MET VAL THR LEU GLU HIS MET SER THR LEU ILE

GAA CTT ACC TCT TGC CAA AGG CCA TTT ATA TTC ATA TAG TGA CAG GCT GTG CTC CAT ATT TTA
TCT CTC ATA GAC CCA GCA CAC TCC AGG CAG CAT CCA CCC CAC AGG CAG AAG AGG ACT GGA CCT 498
SER LEU ILE ASP PRO ALA HIS SER ARG GLN HIS PRO PRO HIS ARG GLN LYS ARG THR GLY PRO

CAG TCA TTT TGG TCA CAA TCG AGG GTT TCT GGA ATT TTC ACA TCC CTT GTC CAG AAT TCA TTC
GCA CCA TCC TCT GAA TGC CAG TGC ATC TTG GGG GAA AGG GCT CCA GTG TTA TCT GGA CCA GTT 561
ALA PRO SER SER GLU CYS GLN CYS ILE LEU GLY GLU ARG ALA PRO VAL LEU SER GLY PRO VAL

CCC TAA GAG TAA TAA TAA ATA ATC TCT AAC ACC AAA
CCT TCA TTT TCA GGT GGG ACT CTT GAT CCA GAG AAG ACA AAG CTC CTC AGT GAG CTG GTG TAT 624
PRO SER PHE SER GLY GLY THR LEU ASP PRO GLU LYS THR LYS LEU LEU SER GLU LEU VAL TYR

AAT CCA GGA CAG AAC CCA GGT CTC CTG ACT CCT GGC CTT CTA TGC CCT CTA TCC TAT CAT AGA 687
ASN PRO GLY GLN ASN PRO GLY LEU LEU THR PRO GLY LEU LEU CYS PRO LEU SER TYR HIS ARG
...
TAA CAT TCT CCA CAG CCT CAC TTC ATT CCA CCT ATT CTC TGA AAA TAT TCC CTG AGA GAG AAC 750
AGA GAG ATT TAG ATA AGA GAA TGA AAT TCC AGC CTT GAC TTT CTT CTG TGC ACC TGA TGG GAG 813
GGT AAT GTC TAA TGT ATT ATC AAT AAC AAT AAA AAT AAA GCA AAT ACC AAA AAA AAA AA 872
                                                                              3'
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Fig. 2. Structures of cDNAs 8-2 and 6-2 and sequence of cDNA 8-2. The restriction map and nucleotide sequence of cDNA E1 (from Merlin *et al.*, 1983) are shown for comparison. (**a**) Restriction map of cDNAs 8-2 and 6-2. The cDNA 5-2 contains the 3′ end of the 8-2 sequence. Restriction sites: Sa, *Sau*3a; M, *Msp*I; Pv, *Pvu*II, Ss, *Sst*I; P, *Pst*I; H, *Hin*dIII; T, *Taq*I; R, *Eco*RI. The 5′ and 3′ ends and sizes in base pairs are indicated. ■, 20-mer oligonucleotide probe sequence. ↓, point of sequence divergence between 8-2 and E1. (**b**) Nucleotide sequence of cDNA 8-2. The amino acid sequence of 8-2 derived from the reading frame giving the longest open coding region is shown. This reading frame also matches that which gives the longest open region in E1. The 20-mer oligonucleotide probe sequence is indicated and the poly(A) addition signals are underlined. ▼, point of divergence between 8-2 and E1. . . . , stop codons.

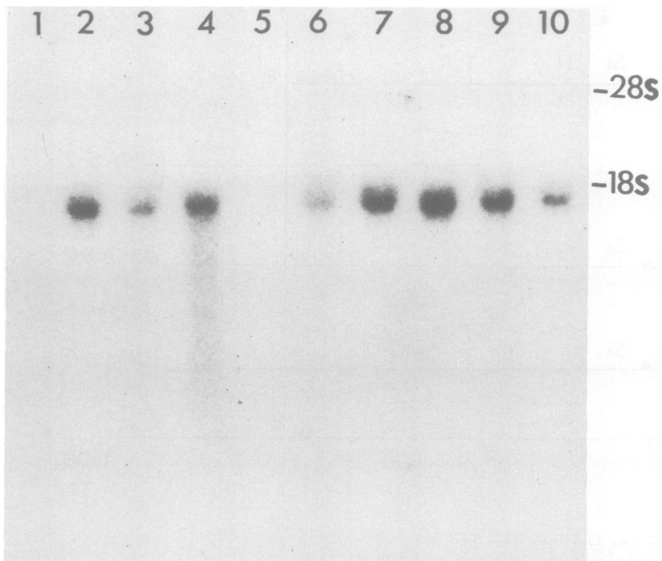

Fig. 3. Size analysis and induction kinetics of 8-2-specific mRNA in T98G and Daudi cells. Total RNA (10 μg) from either Wellferon-induced or untreated T98G or Daudi cells was electrophoresed on formaldehyde-agarose gels and the Northern blots hybridized to nick-translated cDNA 8-2. **Lane 1**, total RNA from untreated T98G cells; **lane 2**, RNA from T98G cells induced for 8 h; **lane 3**, RNA from untreated Daudi cells; **lane 4**, RNA from Daudi cells induced for 21 h. A time course of 8-2-specific mRNA induction in T98G cells is shown in **lanes 5–10. Lane 5**, total RNA (10 μg) from untreated T98G cells; **lane 6**, RNA from T98Gs induced for 2 h; **lane 7**, 4 h induction; **lane 8**, 6 h induction; **lane 9**, 8 h induction; **lane 10**, 24 h induction. rRNA markers of 28S and 18S are indicated.

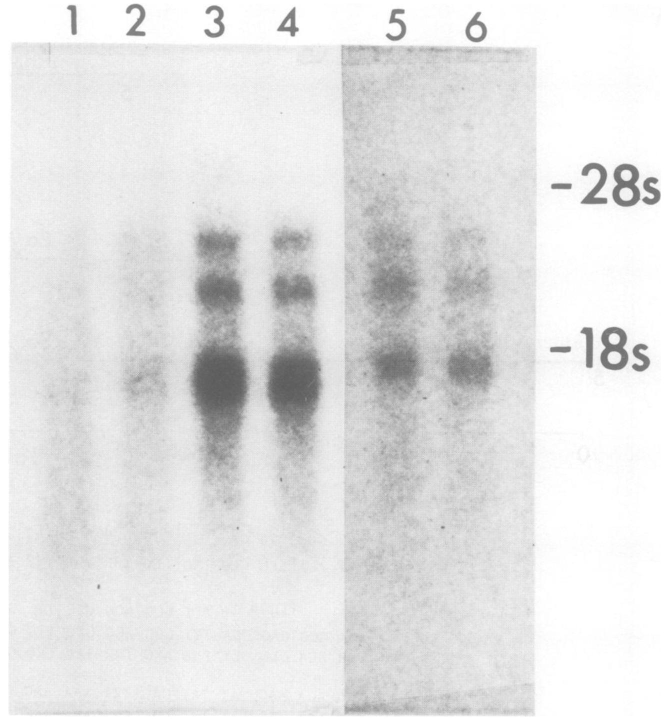

Fig. 4. Analysis of 8-2-specific RNA transcripts in HeLa cells. Total RNA (10 μg) from HeLa cells left untreated (**lane 1**) or treated for 10 h with 1000 U/ml of either IFN-γ1 (Biogen, **lane 2**), IFN-α2 (Shering Corp, **lane 3**) or Wellferon (**lane 4**) were electrophoresed as above and the Northern blots hybridized to nick-translated cDNA 6-2. **Lanes 5** and **6** show **lanes 3** and **4** of the same blot rehybridized to cDNA 5-2. rRNA markers of 28S and 18S are indicated.

Interestingly, no increase in transcripts is seen in RNA from cells treated with IFN-γ1 (Figure 4, lane 2). When the same Northern blot was rehybridized to cDNA 5-2 (Figure 4, lanes 5 and 6), which contains sequences homologous to 8-2 but not E1, only the three largest mRNAs hybridized, suggesting that the 1.5-kb RNA has an identical 5′ end but differs in the 3′ end from 8-2 and 6-2.

The analysis of phage and cosmid genomic clones for the 2-5A synthetase

The isolation of a cDNA with a novel 3′ end sequence and the results of the Northern analysis of IFN-treated HeLa cell RNA suggested that either two 2-5A synthetase genes exist or that a single gene undergoes alternative processing in some cell types. To distinguish between these two possibilities, the genomic human 2-5A synthetase gene specified by hybridization to cDNAs 8-2 and 6-2 was characterized in both phage and cosmid clones. A λ Charon 4A library of a *Hae*III-*Alu*I partial digest of human fetal liver DNA (Maniatis *et al.*, 1978) was screened with cDNA 8-2. The phage isolate that hybridized most strongly (λH2-5A,2) was restriction mapped and subjected to Southern analysis (Figure 5). When digested with *Eco*RI, which excises the genomic insert from the λ arms, six fragments can be identified of approximate sizes 4, 3.5, 2.8, 1.7, 0.8 and 0.6 kb. Each of the *Eco*RI fragments was subcloned into plasmid vectors, restriction mapped individually and compared with the phage map. The 2.8- and 1.7-kb fragments hybridize to cDNA 8-2 (Figure 6b) while cDNA 6-2, which extends 8-2 in the 5′ direction, also hybridizes to the 4- and 3.5-kb *Eco*RI fragments (Figure 6a). The 0.8- and 0.6-kb fragments do not hybridize to cDNA 6-2. In total, at least six exons and five introns can be identified with these cDNA clones.

The extreme 5′ end of the cDNA 6-2 is located in the most 5′ fragment of the genomic phage. However, since 6-2 is still ~300 bp shorter than the estimate of the full-length mRNA provided by the Northern blots of Daudi and T98G RNA, the entire genomic gene may not be contained in λH2-5A,2.

To confirm the genomic phage map, a cosmid library of human genomic DNA was screened with cDNA 6-2. A positively hybridizing clone, pcos2EMBL2-1, was purified and the DNA analysed by Southern blot hybridization. The results (Figure 6c) show that three of the four hybridizing *Eco*RI bands are of identical size to those in phage λH2-5A,2. Hybridization of the cosmid and phage DNA to cDNA 5-2 reveals that the cosmid 6.8-kb band contains the same 3′ end sequences as the phage 1.7-kb band (Figure 6d). Therefore, the apparent differences in the sizes of the hybridizing bands result from the creation of an artificial 3′ *Eco*RI site during the construction of the library.

The genomic site for 2-5A synthetase

The structure of the human 2-5A synthetase gene was examined using genomic blots of high mol. wt. DNA from T98G, Daudi and HeLa cells. DNA (2.5 μg per track) was restricted, electrophoresed on a 0.8% agarose gel and Southern blots hybridized to cDNA 6-2 (Figure 7). An identical pattern of hybridizing bands appeared in DNA from all three cell lines. Furthermore, this pattern was co-linear with that observed in the cosmid DNA analysis: four *Eco*RI bands of 6.8, 4.0, 3.5 and 2.8 kb hybridized. These results indicate that there is a single chromosomal gene for 2-5A synthetase.

Discussion

In these studies, we have isolated and characterized a novel cDNA for the 2-5A synthetase gene from a human cDNA library. Nor-

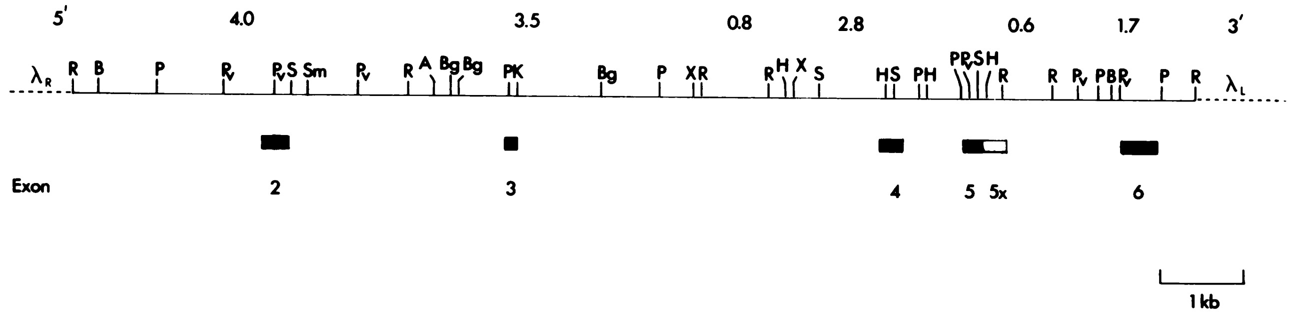

Fig. 5. Restriction mapping of the λH2-5A,2 genomic clone and localisation of the 6-2 cDNA exons. Mapping of the entire phage or of each of the *Eco*RI fragments subcloned into either pBR322 or pSp65 was carried out by standard procedures. Dideoxynucleotide sequencing was using to delinate the exon-intron junctions. The size of each *Eco*RI fragment is indicated above the map, as is the 5′ to 3′ orientation. Note that the genomic insert was oriented in the phage 3′ to 5′ so that the L (left) and R (right) arms appear to be reversed. Exons 2 and 3 were identified in cDNA 6-2; exons 4, 5 and 6 in cDNAs 8-2 or 5-2. Exon 5x represents the extension of exon 5 that constitutes the 3′ end of cDNA E1 (Merlin *et al.*, 1983) and was confirmed by sequencing M13 subclones of the 2.8-kb *Eco*RI fragment of λH2-5A,2. Restriction sites: A, *Ava*I; B, *Bam*HI; Bg, *Bgl*I; H, *Hind*III; K, *Kpn*; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sst*I; Sm, *Sma*I; X, *Xba*I.

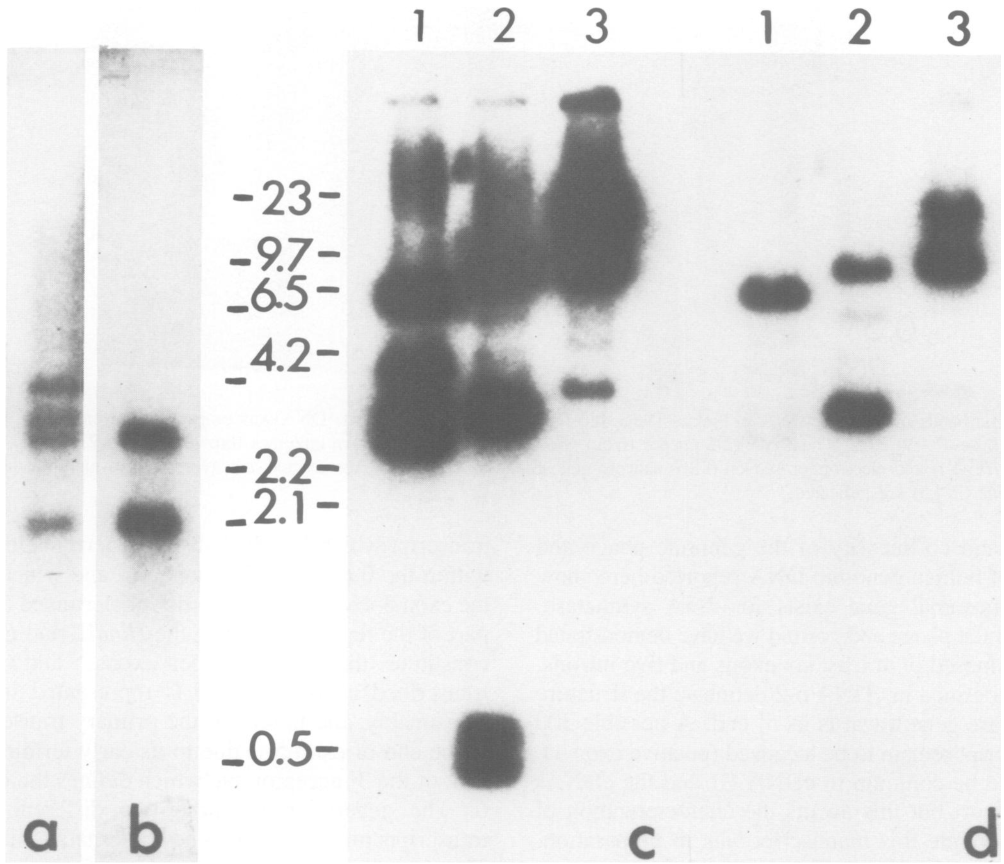

Fig. 6. Comparison of genomic phage, λH2-5A,2 with genomic cosmid pcos2EMBL2-1. **Panels a** and **b**: λH2-5A,2 DNA was digested with *Eco*RI and the Southern blot hybridized to either cDNA 6-2 (**panel a**) or cDNA 8-2 (**panel b**). The 1.7-kb band contains the 3′ end-specific sequences as determined by sequence analysis. **Panels c** and **d**: cosmid pcosEMBL2-1 DNA was digested with *Eco*RI (**lane 1**), *Hind*III (**lane 2**) or *Bam*HI (**lane 3**) and the Southern blot hybridized to nick-translated cDNA 6-2 (**panel c**) or cDNA 8-2 (**panel d**). The 6.8-kb *Eco*RI fragment of the cosmid contains the 3′ end sequences found in the 1.7-kb *Eco*RI fragment of λH2-5A,2. The doublet at ~0.5 kb in the *Hind*III digest (**panel c, track 2**) hybridizes only to cDNA 6-2 (or 8-2) and represents exons 4 and 5.

thern blot analysis and filter selection experiments showed that the cDNA 8-2 specifically binds to an mRNA of approximate size 1.7 kb from both Daudi or T98G cells which is translated into active 2-5A synthetase upon injection into oocytes. This mRNA is of a size sufficient to code for a protein of ~30 000 daltons.

Two forms of 2-5A synthetase enzyme have been described in both mouse and human systems. St. Laurent *et al.* (1983) demonstrated that mouse Ehrlich ascites cells expressed both a large enzyme (85 000 − 100 000 daltons) and a smaller enzyme (20 000 − 30 000 daltons). Using gel filtration and oocyte injection, Revel *et al.* (1981 − 1982) identified enzymes of 60 000 − 80 000 daltons and 30 000 daltons in human SV80 and HeLa cells, whereas Namalwa cells showed only the 30 000 dalton enzyme. It is still unclear whether the two different sizes of 2-5A synthetase enzyme are different proteins or different forms of the same polypeptide and thus derived from a single gene.

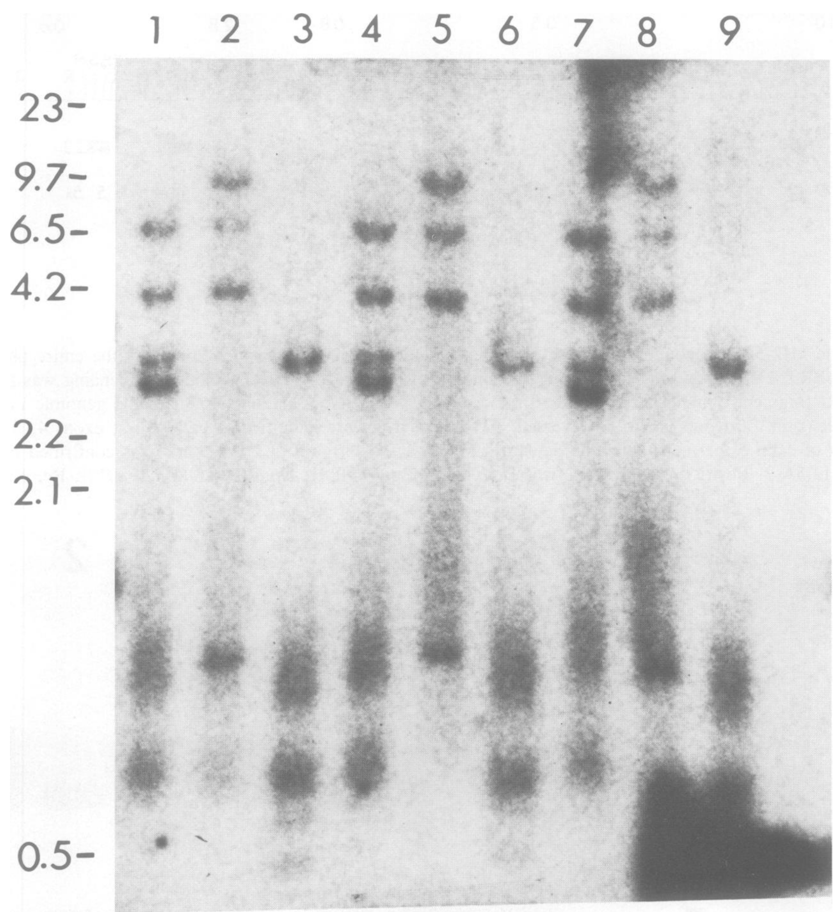

Fig. 7. cDNA 6-2-specific bands of genomic DNA in HeLa, T98G and Daudi cells. High mol. wt. DNA was prepared from either HeLa (**lanes 1–3**), T98G (**lanes 4–6**) or Daudi (**lanes 7–9**) cells. The DNA (2.5 μg per track) was digested with restriction enzymes (**lanes 1, 4 and 7**, *Eco*RI; **lanes 2, 5** and **8**, *Sst*I; **lanes 3, 6** and **9**, *Hin*dIII) and electrophoresed on 0.8% agarose gels. Southern blots were constructed and hybridized to nick-translated cDNA 6-2. Mol. wt. marker positions (in kb) are indicated.

The comparison and co-linearity of the genomic phage and cosmid with blots of human genomic DNA reported here show that a single chromosomal gene exists for 2-5A synthetase. Through mapping of the phage and cosmid we have demonstrated that this gene is composed of at least six exons and five introns.

Exons 2 and 3 as defined in cDNA 6-2 delineate the structure of the 2-5A synthetase gene towards its 5′ end. A possible 300 nucleotides of transcript remain to be localized (putative exon 1). Exons 2 and 3 could be common to cDNA E1 and the cDNAs described in this report but this awaits the characterization of longer E1 cDNAs. While this manuscript was in preparation, a genomic phage map of the 3′ end of the human 2-5A synthetase gene was described by Benech *et al.* (1985). This map includes the 6.8-kb *Eco*RI fragment apparent in the cosmid and genomic blots (Figure 6b) which we have shown to contain the same 3′ end sequences as the 1.7-kb *Eco*RI band in the phage λH2-5A,2 (Figure 6). Further inspection of the λH2-5A,2 map reveals identical restriction sites within the 2.8-kb *Eco*RI fragment and 3.1-kb *Eco*RI fragment described by Benech *et al.* (1985). Located within this fragment are exons 4 and 5. Exon 4 is common to cDNAs 6-2 and E1. Exon 5 is of particular interest since different mechanisms of gene processing must have occurred to generate the E1 and 8-2 mRNAs. In cDNA 8-2, exon 5 comprises ~150 bp, including the *Pvu*II and *Sst*I sites but not the *Hin*dIII or *Eco*RI sites. Exon 6 of cDNA 8-2 (523 bp), is flanked by *Pvu*II and *Pst*I sites and follows a 1.7-kb intron. However, exon 5x in cDNA E1 appears to be derived from a primary transcript which has made use of a termination site located just within the 0.6-kb *Eco*RI fragment, and which fails to splice at the exon 5-exon 6 splice junctions determined in 8-2. As a result, part of the region (including the *Hin*dIII and *Eco*RI sites) which constitutes the intron between exons 5 and 6 in cDNA 8-2 is transcribed as mRNA and is represented in the E1 cDNA. Presumably, the failure of the primary transcript to be spliced at the end of exon 5 is due to its early termination and thus its lack of the 3′ acceptor site which defines the beginning of exon 6. The generation of these two different 2-5A synthetase transcripts may represent a new alternative in RNA processing. The extension of exon 5 to include the 5x region in the E1 mRNA involves a mechanism which is subtly different from other examples of alternative splicing, notably the chicken myosin light chain gene (differential initiation of transcription, Nabeshima *et al.*, 1984), the rat calcitonin-CGRP gene (alternative exon selection, Rosenfeld *et al.*, 1983) and the murine αA-crystallin genes (occasional use of an exon, King and Piatigorsky, 1983).

The results presented here show that the cDNA 8-2 is derived from a 1.7-kb 2-5A synthetase mRNA in Daudi cells which is different in sequence at its 3′ end from the E1 cDNA described previously in SV80 cells. The 6-2 cDNA also identifies a 1.7-kb mRNA in T98G cells and four mRNAs of sizes 1.5, 1.7, 2.5 and 3.5 kb in HeLa cells. The smallest of the HeLa mRNAs was found to differ in sequence at the 3′ end from the larger mRNAs since it failed to hybridize to cDNA 5-2 which contains sequences solely represented in cDNAs of the 8-2 type. It is possible that both the E1 and 8-2 cDNAs are synthesized in HeLa cells as products of alternative RNA processing as described above. The reason why two forms of a small enzyme activity should be required in some cells (HeLa) but not in others (T98G) or how this regulation is achieved remain to be determined. In addition, the structural analysis of the 2.5- and 3.5-kb HeLa transcripts has yet to be completed, although it is clear that they both contain 3′ sequences homologous to 8-2 (Figure 5). Both of these mRNAs are adequate in length to encode the 85 000-dalton 2-5A synthetase described in HeLa cells (Wells *et al.* 1984). However, they may also represent incompletely processed transcripts. This can only be resolved by further characterization.

The cDNA 6-2 is ~1400 bp and, since it does not contain an initiator ATG, it is unlikely to encode a complete 2-5A synthetase mRNA. The 5′ end of 6-2 is located in the middle of the 5′ (4.0 kb) *Eco*RI fragment of the phage clone λH2-5A,2. Further studies of this fragment and of the cosmid clone to locate the 5′ end of the 2-5A synthetase gene are currently under way.

Materials and methods

cDNA and genomic libraries

The Daudi cDNA library was constructed as described (Friedman *et al.*, 1984) from human Daudi cells induced with 500 U/ml Wellferon (human α-IFNs, 2 x 10^8 IU/mg protein, Burroughs Wellcome Co.) for 18 h. The human genomic phage library (from T.Maniatis) was constructed using partial *Hae*III-*Alu*I digests of fetal liver DNA inserted into the λphage Charon 4A using *Eco*RI linkers (Maniatis *et al.*, 1978). The human genomic cosmid library, h2a (from A.Frischauf), contained DNA partially cleaved with *Mbo*I and ligated into the *Bam*HI site of pcos2EMBL. This library was plated on *E. coli* strain BHB3175 (Poustka *et al.*, 1984) and screened with cDNA 6-2.

Screening with oligonucleotide probes and cDNAs

A group of eight 20-mer oligonucleotide probes was predicted from a deduced amino acid sequence of the 2-5A synthetase (Chebath *et al.*, 1983) and generously synthesized by N.Stebbing (AMGen Inc.). The probe sequences were: 5′ AC A/G AA A/G TTC TTA ACC CT A/G CC 3′. For end-labelling, 100 pmol of mixed 20-mer probes (0.64 μg total) were combined with 100 mM Tris-HCl pH 8, 50 mM $MgCl_2$, 5 mM dithiothreitol (DTT) in a tube in which 150 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) has been dried down. 2 μl of T4 kinase (Bethesda Research Laboratories) were added and the reaction allowed to proceed for 45 min at 37°C. The reaction was stopped by heating for 10 min at 65°C followed by passage over Sephadex G-50. Probes were routinely labelled to 2–4 x 10^8 c.p.m./μg and 1.5 x 10^8 (6 x 10^6 c.p.m./ml) was used for the initial library screen. Hybridizations using the 20-mer probes were carried out at 37°C in 5 x SSC, 5 x Denhardt's, 0.05% Na pyrophosphate and 100 μg/ml calf liver tRNA. The most stringent wash was done in 1 x SSC, 0.1% SDS for 15 min at 47°C. Screening of phage and cosmid libraries with nick-translated cDNAs was carried out as described for Southern blot hybridizations (see below) with the most stringent wash at 0.2 x SSC, 0.1% SDS for 30 min at 65°C.

Preparation of total and poly(A)$^+$ RNA

T98G cells were induced for 8 h with IFN (as detailed in Figure legends), Daudi cells for 21 h and HeLa cells for 10 h. Total RNA was prepared essentially as described by Chirgwin *et al.* (1979). The RNA was used directly as total RNA or passed over oligo(dT)-cellullose (Collaborative Research) to obtain the poly(A)$^+$ fraction.

Oocyte injection and 2-5A synthetase activity assay

Xenopus laevis frogs were maintained, oocytes obtained and microinjection carried out as described by Gurdon and Melton (1981). Groups of 10–20 defolliculated oocytes were injected with a total of 0.5–1.0 μg poly(A)$^+$ RNA (50 nl/oocyte of RNA at 0.5–1.0 μg/ml) and incubated overnight at 25°C in 0.5 ml Barth's buffer (Gurdon, 1968). The oocytes were homogenized in 300 μl of lysis buffer (20 mM Hepes pH 7.5, 10 mM KCl, 5 mM Mg acetate, 1 mM DTT, 0.5% NP 40), lipid layers removed by suction and 20 μl of the extract bound to poly (rI.rC) agarose beads for 2-5A synthetase assay. The assay was exactly as described (Williams *et al.*, 1981; Penn and Williams, 1984) except that 4 μCi/assay of fresh [γ-^{32}P]ATP (400–600 Ci/mmol, Amersham Inc.) was used as the substrate and the assay volume was reduced to 20 μl. Enzyme reaction products were treated with bacterial alkaline phosphatase as described (Williams *et al.*, 1981) followed by spotting 5 μl on polyethylene iminecellulose t.l.c. plates (Polygram Cel 300 PEI, Macherey). Chromatography was carried out in 1.0 M acetic acid and the t.l.c. plates autoradiographed (Williams *et al.*, 1981).

Filter selections

Subcloning of the cDNA 8-2 into pUC 9 and filter selections were done using established techniques (Maniatis *et al.*, 1982) with some modifications. For filter selection, 20 μg of either pMS 8-2 or pUC 9 DNA was bound to 3 mm nitrocellulose circles by repeated spotting and drying. Selection was carried out by adding the filter to a tube in which 20 μg poly(A)$^+$ RNA had been dried down containing 300 μl 65% deionized formamide, 20 mM Pipes (piperazine-N,N′-bis[2-ethane sulfonic acid]) pH 6.4, 0.2% SDS, 0.4 M NaCl, 100 μg/ml calf liver tRNA and 20 μg/ml oligo(A)$^+$. Hybridization was at 70°C for 10 min followed by 3 h incubation at 50°C. After extensive washing at 65°C in 10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA and 0.5% SDS (the final two washes did not contain SDS), selected mRNA was eluted by boiling in DEP-treated water. The mRNA was extracted with phenol:chloroform (25:24) and ethanol precipitated before injection into oocytes.

Northern blots

Total RNA (10 μg) or poly(A)$^+$ RNA (1.5 μg) samples were denatured in 50% formamide, 6.7% formaldehyde in MOPS buffer [400 mM MOPS (3-[N-morpholino]propane sulfonic acid) pH 7, 100 mM Na acetate, 10 mM EDTA]. After heating for 10 min at 65°C, samples were electrophoresed on a 1.5% agarose gel containing 6.4% formaldehyde in MOPS buffer. Blots were done on Zetaprobe (Biorad) in 20 x SSC. Hybridization to nick-translated probes was carried out at 37°C in 50% formamide, 5 x SSC, 5 x Denhardt's, 0.1% SDS and 150 μg/ml denatured salmon sperm DNA. The most stringent wash was in 0.2 x SSC, 0.1% SDS for 30 min at 50°C.

Genomic blots

High mol. wt. DNA was obtained from T98G, Daudi and HeLa cells by using a rapid procedure as follows. Approximately 1-8 x 10^7 cells were lysed in 75 mM NaCl, 24 mM EDTA, 1% SDS. Solid proteinase K was added to 0.2 mg/ml and incubated for 2.5 h at 37°C. The viscous DNA was gently extracted 3 times with phenol, 3 times with chloroform:isoamyl alcohol (24:1) followed by ethanol precipitation. Restriction digests were done on 2.5 μg of DNA at 37°C for 18 h. DNA was electrophoresed on 0.8% agarose gels in 0.089 M Tris-borate pH 8.3, 20 mm EDTA for 5 h, at 30 mA. Southern blots were done in 10 x SSC and hybridization to nick-translated probes carried out as described for Northern blots except that the most stringent wash was in 1 x SSC, 0.1% SDS at 65°C for 30 min. For cosmid and genomic phage blots, 3–5 μg DNA was restricted and Southern blotting and hybridization done as described above except the most stringent wash was at 0.1 x SSC, 0.1% SDS at 65°C for 30 min.

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